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I. CONTROL MICROBIANO DE INSECTOS CON PROTOZOOS

Coordinador: **Carlos E. LANGE**

Strategies for evaluating the host specificity of Lepidopteran Microsporidian

SOLTER, Leellen F. and Joseph V. MADDOX
Illinois Natural History Survey, 607 E. Peabody Dr., Champaign, IL 61820, USA.

■ **ABSTRACT.** The host specificity of insect pathogens being considered for release as classical biological control agents is difficult to measure prior to release into the target pest population. This is because host specificity data generated in the laboratory may not accurately predict the host specificity of biological control agents in the field. Laboratory (physiological) host range of most insect pathogens is usually far broader than the ecological host range. Laboratory host range studies were designed to test this contention and to more accurately predict ecological host specificity. The host specificity of five species of microsporidia that are endemic in gypsy moth (*Lymantria dispar*) populations in Europe but are not found in North American populations were tested in nontarget hosts. Half of the 49 native species of forest Lepidoptera tested were infected by these isolates, yet the microsporidia did not develop optimally in the nontarget hosts. We then fed viable spores of microsporidia isolated from nine species of North American forest Lepidoptera to *L. dispar* larvae. All of these microsporidia infected *L. dispar*, yet, despite a 130-year history of host sympatry in North American forests, they have not been recovered from *L. dispar* in North America. We observed the same types of atypical infections in *L. dispar* infected with the native microsporidia that were observed when *L. dispar* microsporidia were fed to native nontarget hosts. We are attempting to determine the ecological level at which the microsporidia exhibit host specificity using horizontal transmission studies. Simple but ecologically more complex bioassay experiments may be useful in predicting ecological host specificity. In addtion, the biological control literature suggests that information about the host specificity of potential biological control agents should be collected in the aboriginal range. We surveyed the pathogens of nontarget lepidopteran populations from areas in Bulgaria where three microsporidian genera are endemic in *L. dispar* populations. We are currently evaluating the potential for these microsporidia to infect *L. dispar* and attempting to determine whether the microsporidia found in nontarget Lepidoptera are the same species as those found to be endemic in the *L. dispar* populations.

■ **RESUMEN. Estrategias para evaluar la especificidad hospedadora de microsporidios de lepidópteros.** La especificidad hospedadora de patógenos considerados para ser liberados como agentes de control biológico clásico es difícil de medir antes de la liberación del agente, pues los datos sobre especificidad generados en el laboratorio pueden no llegar a predecir con exactitud la especificidad de los agentes en el campo. El espectro hospedador de laboratorio (fisiológico) de la mayoría de los entomopatógenos es mucho más amplio que el ecológico. Nuestros estudios sobre espectro hospedador fueron di-

señados para testear este punto de vista. Primero probamos la especificidad de cinco especies (en tres géneros) de microsporidios endémicos en poblaciones de la "polilla gitana" (*Lymantraia dispar*) de Europa pero que no se encuentran en las poblaciones de América del Norte. Teniendo cuidado de seleccionar especies hospedadores "no objetivos" que superponen su distribución con poblaciones de *L. dispar* en los bosques estadounidenses, encontramos que más de la mitad de las 49 especies de lepidópteros de bosques testeados fueron infectados con estos aislamientos, aunque los microsporidios no mostraron un desarrollo óptimo en los hospedadores "no objetivos". Luego administramos esporos viables de microsporidios aislados de nueve especies de lepidópteros de bosques de América del Norte a la "polilla gitana". Todos estos microsporidios infectaron a *L. dispar*. Sin embargo, a pesar de 130 años de simpatría en los bosques de América del Norte, ellos no han sido nunca aislados de *L. dispar* en las amplias prospecciones realizadas. Observamos el mismo tipo de infecciones "atípicas" en *L. dispar* infectados con microsporidios nativos como las que observamos cuando microsporidios de *L. dispar* fueron administrados a hospedadores nativos "no objetivos". Estamos tratando de evaluar nuestras predicciones de dos maneras. En un esfuerzo para determinar el nivel ecológico al cual los microsporidos exhiben especificidad, confinamos larvas de *L. dispar* infectadas con microsporidios junto con larvas sanas en los mismos sustratos en el laboratorio. Tres de los nueve microsporidios fueron transmitidos de larvas infectadas a sanas. Cuando se aumentó la complejidad ecológica de los recipientes comunes mediante la colocación de larvas infectadas y sanas sobre follaje cubierto por "una fina malla" no ocurrió transmisión. Así, bioensayos experimentales simples pero ecológicamente más complejos pueden ser de utilidad para predecir el espectro hospedador ecológico. La bibliografía sobre control biológico sugiere que la información acerca de la especificidad hospedadora de potenciales agentes de control biológico debería ser obtenida en el area de distribución nativa. Nuevas tecnologías, combinadas con nuestra experiencia en la evaluación de infecciones microsporidianas en "no objetivos" en el laboratorio, deberían permitirnos determinar si los microsporidios encontrados en poblaciones "no objetivos" son "coespecíficos" con las especies de *L. dispar*. Con este propósito hemos explorado los patógenos de poblaciones de lepidópteros "no objetivos" en zonas de Bulgaria, donde tres géneros de microsporidios son endémicos en poblaciones de *L. dispar*. Cuatro microsporidios fueron encontrados en hospedadores tortrícidos y noctuidos, los cuales estaban consumiendo las mismas plantas hospedadoras que las larvas de *L. dispar*. En la actualidad, estamos evaluando el potencial de estos microsporidios para infectar a *L. dispar*. También estamos intentando determinar si estos microsporidios constituyen las mismas especies que las encontradas endémicamente en las poblaciones de *L. dispar*.

INTRODUCTION

Host specificity studies are essential for scientifically-based regulatory decisions regarding the introduction of exotic organisms for biological control programs. In addition, they are important for understanding the mechanisms that give rise to host range expansion, evolution, and diversity of pathogens (Cate & Maddox, 1994). Nevertheless, evaluating the results of host specificity testing in the laboratory may be difficult when the laboratory data must be interpreted for a field situation.

The ecological host specificity (host specificity in the field) of biological control agents may not be accurately predicted by physiological host specificity data generated in the laboratory. Many insect pathogens, including the virus, fungi, and microsporidia (Protozoa) groups, appear to have

broad host ranges under typical laboratory conditions and unusual exposure routines (Fisher & Sanborn, 1962; Undeen & Maddox, 1973; Fournie *et al.*, 1990; Hajek *et al.*, 1995), but pathogen surveys of selected natural hosts indicate that many of these pathogens are probably quite host specific (Campbell & Podgwaite, 1971; Nordin, 1971; Andreadis *et al.*, 1983; Siegel *et al.*, 1988; Jeffords *et al.*, 1989; Hajek *et al.*, 1996). Laboratory host specificity testing of insect pathogens has traditionally followed the methodology of chemical insecticide testing. Selected nontarget hosts are exposed to high dosages of pathogens in confined arenas. These bioassays produce only "susceptible" or "not susceptible" results. This type of testing and predictions based on these results are useful if inundative releases of microbial pesticides are planned, but testing methods probably should be modified and the results interpreted differently when pathogens are to be inoculatively or augmentatively released as classical biological control agents.

Microsporidia. The Phylum Microsporidia is considered to be a unique group of protistans. Approximately 1000 species have been described, primarily from insects and other arthropods in which they are most commonly found. Microsporidia are obligate pathogens and develop in the cytoplasm of host cells; the tissues which are primarily infected (target tissues) differ with the species of microsporidia. Infection is horizontally transmitted when hosts ingest viable, infective spores, or vertically when an infection is transmitted from an infected female to her offspring, either on the surface of or within the eggs. Many microsporidian species are capable of both horizontal and vertical transmission.

Some microsporidia are relatively virulent and kill the larval host prior to or during pupation but the most common effect on hosts is chronic debilitation. Life span and fecundity of adults is frequently reduced and transovarial transmission often increases larval mortality (Brooks, 1988).

These effects often lead to suppression of host populations (Stairs, 1972; Siegel *et al.*, 1988).

***Lymantria dispar* and control efforts.** *Lymantria dispar*, the gypsy moth, was accidentally introduced in the U.S. in 1868 and was recognized as a serious forest tree defoliator, as well as a serious pest in suburban areas (McManus & McIntyre, 1981), within 20 years of introduction. The insect prefers to feed on the leaves of oak trees but over 100 species of trees and shrubs are included in its host plant range (Leonard, 1981). Efforts to control *L. dispar* have included the use of chemical insecticides, particularly Dimilin, *Bacillus thuringiensis kurstaki*, and biological control agents such as carabid predators and several species of dipteran and hymenopteran parasitoids.

Recently, concerns about the effects of chemicals and Bt insecticides on nontarget insects in large areas of contiguous forest have led to increased interest in use of classical biological control methods. The U.S. Forest Service produced a nuclear polyhedrosis virus for use as a microbial insecticide. A fungal pathogen, *Entomophaga maimaiga*, has recently been manipulated in areas at the leading edge of *L. dispar* dispersal and has decimated *L. dispar* populations in some areas (Hajek & Elkinton, 1993).

Nonetheless, *L. dispar* populations in North America continue to outbreak more frequently and attain higher population densities than in Europe where the pest evolved with a complex of natural enemies. Previous reports have indicated that *L. dispar* populations are influenced by the presence of several biotypes of microsporidia (Weiser & Novotny, 1987), and scientists from Central Europe and the U.S. are currently cooperating in studies on this group of pathogens, including taxonomy, ecology, physiology, epizootiology, and host specificity testing.

Host specificity testing. We designed laboratory host range studies to test the host specificity of several microsporidian species parasitizing forest Lepidoptera (Solter *et al.*, 1997). Our evaluations of host specificity were based on several criteria: 1. how the pathogens were to be used (inundative or inoculative release), 2. the ecological relevancy of the nontarget hosts to be tested to the system into which the potential biological control agent will be released, and 3. how the infections in the nontarget hosts compared to infections in the natural (or target) host. We also evaluated the adequacy of laboratory experiments to predict the ecological host range of these insect pathogens using additional laboratory experiments (Solter & Maddox, 1998), and are currently attempting to validate our predictions of ecological host range in the field.

Host specificity of *L. dispar* microsporidia. We tested the host specificity of five biotypes of mi-

crosporidia that are endemic in *L. dispar* populations in Europe but are not found in North American populations (Solter *et al.*, 1997). We selected nontarget host species that occur sympatrically with *L. dispar* populations in the U.S. forests. Over half of the 49 species of forest Lepidoptera we tested developed infections, however, the microsporidia did not develop optimally in the most of the nontarget hosts. The responses of the nontarget insects to the microsporidia were categorized to reflect the risk we predicted to nontarget populations (Table I).

The categories of infection were given the labels "host-like", "heavy", "atypical" and "refractory". We defined host-like infections as those in which the microsporidian infection could not be differentiated from infection in the natural host and for which similar rates of infection were observed. Heavy infections were those in which environmental spores that appeared to be viable were produced in the nontarget hosts, but one or more of several "nontarget responses" were observed: few larvae became infected of those fed spores, significantly lower numbers of environmental spores were produced, many spores were atypical in appearance, and host cellular immune response in the form of tissue melanization was observed. Infections categorized as atypical included the above nontarget responses but few or no environmental spores were produced. No infection was observed when larvae were categorized as refractory and larval development was normal.

We predicted that the nontarget species that developed host-like infections were at risk and species that developed atypical infections or were refractory to the microsporidia were not at risk for inoculative releases. Nontarget species with infections designated as heavy were difficult to assess because, although the microsporidia did not develop optimally, the production of environ-

Table I. Host ranges of *L. dispar* microsporidia.

Microsporidia Biotypes	Host-like infection	Heavy infection	Atypical infection	No Infection
Nosema portugal[a]	2	8	16	22
Nosema sp. [Romania biotype]	5	9	5	7
Nosema sp. [Slovakia biotype]	2	5	2	7
Vairimorpha sp. [Czech biotype]	4	19	8	6
Endoreticulatus [Portugal biotype]	15	3	2	13

[a] Maddox *et al.*, 1999.

Table II. Results of direct feeding of forest Lepidoptera microsporidia to *L. dispar*; three most infective species of nine species tested.

Host/Microsporidium	% Infection 10^5 (n)
Malacosoma americanum Nosema sp.	
M. disstria	93.0 (43)
L. dispar[a]	91.7 (48)
Hyphantria cunea Vairimorpha sp.	
H. cunea	92.5 (40)
L. dispar[a]	83.9 (31)
Hyphantria cunea Endoreticulatus sp.	
H. cunea	100 (40)
L. dispar[a]	89.7 (58)

[a] Laboratory host.

mental spores suggested that it would be possible for the pathogen to be transmitted within the nontarget host population. The *Endoreticulatus* biotype produced host-like infections in nearly half of the nontarget hosts to which it was fed and will not be considered for release in the United States.

Evaluations of laboratory host specificity testing. We performed several bioassays in the laboratory to study the significance of the atypical responses of nontarget hosts to microsporidia (Solter & Maddox, 1998). Because we were unable to obtain more Lepidoptera in numbers needed for bioassays, we examined a "reverse" system. *L. dispar* was introduced into North America approximately 130 years ago and, although microsporidia naturally infect several species of forest Lepidoptera with which *L. dispar* is sympatric, microsporidia have never been recovered from extensive surveys of *L. dispar* pathogens in the U.S. (Campbell & Podgwaite, 1971; Podgwaite, 1981; Andreadis *et al*, 1983; Jeffords *et al.*, 1989). We isolated microsporidia from nine species of North American forest Lepidoptera and fed the viable spores to *L. dispar*. All of these microsporidia infected *L. dispar* with the same range of responses we observed when we fed European *L. dispar* microsporidia to native lepidopterans (Table II). Although no host-like infections occurred, all nine microsporidian species produced environmental spores in *L. dispar*. We predicted that these suboptimal infections would not be transmitted within nontarget populations.

At what level of ecological complexity do the microsporidia exhibit host specificity? To determine if infection was due to the unusual conditions in the laboratory (crowding, forced ingestion of spores), we placed *L. dispar* larvae infected with native microsporidia into confined laboratory arenas with uninfected *L. dispar* larvae. Three of the nine microsporidian species were transmitted from infected to uninfected larvae but in much lower numbers than between the natural hosts. When the ecological complexity of the common arenas was further increased by placing the infected and uninfected larvae on sleeved host plant foliage, no transmission occurred (Table III). These results indicate that simple but ecologically more complex bioassay experiments may be useful in predicting ecological host specificity.

These horizontal transmission studies represent ecologically more realistic exposures to microsporidian spores than do bioassays in which large quantities of viable spores isolated from the natural host are fed to individual nontarget hosts as a "maximum challenge". These experiments and the field data reporting that these microsporidia are not found in U.S. populations of *L. dispar* suggest that observable differences between infections in the natural host and nontarget hosts indicate physiological and ecological barriers to horizontal transmission in field populations of the nontarget hosts.

Field evaluation of host specificity predictions. The predictions we made regarding the host specificity of the *L. dispar* microsporidia could be evaluated in the field in two ways. One method involves release of the microsporidia into *L. dispar* populations in the United States and monitoring of sympatric nontarget populations. A second option is to gather information about the host specificity of potential biological control agents in the aboriginal range. At the present time, we do not have permission to release the pathogens into large plots or contiguous forest areas in the United States. Therefore, as a field evaluation of our host range predictions, we surveyed the pathogens of nontarget lepidopteran populations from several areas in Eastern and Central Bulgaria.

Table III. Larva to larva transmission of microsporidia in natural hosts and nontarget hosts.

Microsporidia	host - host #inf/#exp[a]	host - L.d.[b] #inf/#exp in cups	L.d. - L.d. #inf/#exp. in cups	L.d. - L.d. #inf./#exp. on plants
M. americanum Nosema sp.	15/25	0/25	1/65	0/50
H. cunea Vairimorpha sp.	9/25	0/25	4/65	0/50
H. cunea Endoreticulatus sp.	15/25	13/24	2/60	0/50

[a] number infected/number exposed.
[b] *Lymantria dispar*

Three microsporidian species in the genera *Endoreticulatus, Nosema,* and *Vairimorpha* are endemic in *L. dispar* populations in Bulgaria, one in each of the three study sites we chose (Pilarska *et al.*, in press). We found four microsporidian biotypes parasitizing tortricid and noctuid hosts which were feeding on the same host plants with *L. dispar* larvae (Table IV).

We tested these microsporidia for capability to infect *L. dispar* and have determined that the microsporidia are not the same species as those found to be endemic in the *L. dispar* populations. Table V lists the microsporidia that occurred in each study site in *L. dispar* and in the nontarget Lepidoptera we collected. The microsporidian genera found were different for *L. dispar* and the nontarget Lepidoptera in each site.

We isolated microsporidia from the nontarget species and fed the mature spores to *L. dispar* (Table VI). One *Vairimorpha* sp., found in a dead tortricid larvae, probably *Archips xylosteana*, produced infection in a small number of *L. dispar* and *Spodoptera exigua* larvae. One *L. dispar* larva developed a heavy infection and spores were infective to other *L. dispar* larvae, but the spores were larger than those that were produced in three other lightly infected larvae and were probably a different species.

CONCLUSIONS

Although data from previous field collections suggested that microsporidia were relatively host specific, results from traditional laboratory studies interpreted in the same way as insecticide bioassays suggested that microsporidia have broad host ranges. When we fed microsporidian spores to nontarget hosts in the laboratory, infections were produced in a large number of nontarget host species, however, when more ecologi-

Table IV. Microsporidia recovered from nontarget populations in Bulgaria.

Site (# collections)	Nontarget hosts # infections/#individuals (# spp.)
Levishte, Northwest mountains (3)	0/52 (25+)
Asenovgrad, central plain (2)	3/113 (25)
Kozhuch, Southwest hills	0/175 (45+)
Melnik, Southwest mountains	6/243 (50+)

Table V. Identification of microsporidia from *Lymantria dispar* and nontarget Lepidoptera in Bulgaria.

Site	*L. dispar* microsporidia	Nontarget microsporidia
Levishte	*Nosema* sp.	none found
Asenovgrad	*Endoreticulatus* sp.	*Nosema*–type, *Vairimorpha*-type
Kozhiuh	*Vairimorpha* sp.	none found
Melnik	none found	*Nosema*-type, *Vairimorpha*-type

Table VI. Infectivity of bulgarian nontarget microsporidia to *Lymantria dispar*

Site	Microsporidian genus	Infectivity to *L. dispar*	Infectivity to *Spodoptera exigua*
Asenovgrad	*Vairimorpha* sp. 1	0/10	0/17
	Vairimorpha sp. 2	0/23	0/17
	Vairimorpha sp. 3	4/25	2/16
Melnik	*Nosema* sp.	0/31	0/24

cally realistic bioassays were conducted, the results were very different. Laboratory studies are often the only practical way to evaluate host specificity and we therefore developed some simple methods that better predict ecological host range. These methods include comparison of the progression of infection in the natural and nontarget hosts, larva to larva transmission bioassays, and use of established, nonindigenous pests as models for testing hypotheses about pathogen groups. Additionally, we evaluated these methods in the field using historical data for *L. dispar* as a model nontarget host, and studied *L. dispar* and the range of its microsporidian pathogens in the aboriginal area.

Experimental protocols that stringently test the host range of pathogens using an ecologically rational selection of nontarget hosts are likely to produce results that predict the ecological host range of potential biological control agents more accurately, establish regulatory guidelines that are scientifically based, and supply the information needed to make more informed decisions regarding release of biological control agents.

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